THE MOUSE CIGUATOXIN BIOASSAY: DIRECTIONS FOR USE TO CONTROL FISH FOR CONSUMPTION

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Ciguatera fish poisoning causes serious health problems around the world but the diversity and heterogeneity of ciguatoxins are delaying chemical and immunological remedies. Realistic methods for ciguatoxin screening of fish are needed for public health studies. The mouse bioassay may prove useful since it is simple and relatively cheap. Qualitative and semi-quantitative analyses for ciguatoxins from 50, 100 or 200g of fish tissue using respectively 2, 6 or 12 animals are precisely described.

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Ciguatera is a human illness, sporadic in nature, caused by the ingestion of a wide variety of fish typically associated with coral reefs. These fish are transvectors for multiple ciguatera toxins (mainly ciguatoxins) acquired through their diet (Vernoux & Abbad el Andaloussi,1986; Legrand et al.,1990; Lewis & Sellin,1992). Fish poisoning is present in many coral reef areas of the world (Bagnis,1981) with 10,000–50,000 persons affected each year. Its incidence has been estimated at 1–4/000 population in the Pacific area (Bagnis,1979; Lewis,1984; Yasumoto et al.,1984), 4.2/000 in the Virgin Islands (Olsen et al.,1984), and 3–10/000 at Saint Barthelemy Island (Vernoux, this memoir) and in the Saintes Islands (Czernichow et al.,1984). With the increasing use of air travel, tropical reef fish or consumers (inhabitants or tourists) are constantly moving and ciguatera is being documented in temperate regions (Lange et al.,1992). Thus, ciguatera is a world health problem and there is a need for a practical screening method for ciguatera toxins. Amounts of ciguatoxin in fish that pose a public health problem are very low. One approach to the estimation of dangerous levels of toxin in fish tissue is to dose it in those portions that had elicited human ciguatera poisoning. Some authors did this using mouse or mosquito bioassays (Yasumoto et al.,1984; Chungue et al. 1984; Bagnis et al.,1987; Vernoux, this memoir). Assuming that 500ng CTX can kill c.1000g of mouse (i.p. LD_{50} of CTX into mice=0.45μg/kg), these studies show that the minimum threshold for the human pathogenic dose is c.50–100ng. This level corresponds to 100–200g of mouse killed by a 200g fish portion i.e. the flesh sample has a specific toxicity of 0.5–1g of mouse killed per g of flesh). So only trace quantities of ciguatoxins are needed to elicit human poisoning (0.25ppb). Therefore only those detection methods capable of detecting as little as 250–500pg of CTX per gram of fish flesh need be considered. HPLC and immunological methods could be appropriate methods. Unfortunately, until now the detection of these extremely low levels of multiple ciguatoxins (>20, Legrand et al.,1990) has delayed the use of HPLC methods. Immunological methods that possess the desired sensitivity and specificity have already been developed (Hokama,1990; Park et al.,1992). However, the potential of these methods for large scale use remains to be demonstrated, at least in part due to the possibilities of multiple ciguatoxins in fish contamination. A biological assay which encompasses all the different toxins and gives total toxicity could be used as a robust method for public health control of fish. Two bioassays having the desired sensitivity are the mosquito injection bioassay (Chungue et al., 1984) and the mouse injection bioassay (Hoffman et al.,1983). The latter is preferred since it is more convenient, simple, specific and has been widely used. After having used it for 20 years, we present some recent developments in its use as screening method.

METHODS

Flesh Extraction

Raw or cooked minced flesh can be used since ciguatoxins are heat resistant. If cooking, use a boilable cooking pouch filled with raw minced sample and boil in water for 30 minutes.

A typical procedure to extract 50g of flesh is:

TABLE 1. Testing of fish for consumption by the mouse bioassay
1, prepare LR for 50g of flesh and dilute with 2ml of 1% Tween 60 saline at 37°C and homogenize thoroughly.
2, inject i.p. into 2 male mice (18–24g) at dosage d=0.04ml/g of mice (i.e. 1g equivalent of flesh per gram of mouse).
3, Observe symptoms during 4hr and conclude for ciguatoxin presence (=penile erection) for neurotoxin presence (=respiratory distress) or for okadaic acid or fatty acid presence (crawling gait, slow breathing and general cyanosis).
4, note death after 24hrs and weigh the survivors. Use the following table to indicate edibility.

Observed mortality after a 1g.eq. of flesh injection/g of mouse	Toxin concentration in flesh	Loss of weight at 24hr (>5%)	Interpretation
2/2	≥ 1MUg/g.e.f	-	not edible
1/2	0.5 to 1 MUg/g.e.f	yes	not edible
0/2	<0.5 MUg/g.e.f	yes no	bordeline edible

1, homogenise 3 min in a Waring blender with 150ml of acetone, 2, filter onto a buckner funnel and wash the remaining cake with 30ml of 80% acetone; discard the cake, 3, remove acetone on a rotary evaporator under reduced pressure and reduce the volume of the remaining aqueous solution to 30ml (add water if below), 4, add 10ml of ethanol, shake and extract twice with 40ml of diethyl ether, 5, remove diethyl ether and residual water under reduced pressure (addition of ethanol allows to remove quickly residual water), 6, dissolve the diethyl ether residue in 25ml of 80% methanol and wash twice with 50ml of hexane; discard the hexane solubles, 7, remove methanol and water under reduced pressure; resulting dry residue is called lipid-soluble residue (LR), 8, if weight of LR is >75mg dissolve in 10ml of 80% methanol and wash again twice with 20ml of hexane, 9, emulsify LR in 2ml of 1% Tween 60 saline and keep at –20°C until use.

Determination of Toxin Concentration

LR emulsified in 1% Tween 60 saline was heated at 37°C and i.p. injected into male mice weighing 18–24g (2 mice per dose). A series of doses that vary in a geometric progression by a factor of 1.1938 are assayed. Doses are expressed in gram equivalents of flesh per gram of animal (g.e.f/g). They were chosen in succession in the following series of numbers running from 10^{n-1} to 10^{n} which increase successively by a constant power of 10 (since $1.1938^{13} \times 10^{n-1} = 10^{n}$). Fourteen numbers in such a series are 0.10, 0.12, 0.14, 0.17, 0.20, 0.24, 0.29, 0.35, 0.41, 0.49, 0.59, 0.70, 0.84, 1.0. One gram equivalent corresponds here to 0.04 ml of LR solution and the volumes to be injected are respectively: 0.04 ml/g of mouse multiplied by the numbers indicated in this series i.e. 0.004 ml/g; 0.0048 ml; 0.0056 ml; 0.0068 ml, etc. Note that the total weight of mouse to be injected requires a total volume below that available in the experiment and the use of mice <20g for the highest dosage may be necessary.

Doses <1g.e.f/g are injected in 0.8ml per 20g mouse by carrying out dilutions directly in the syringe with 1% Tween 60 saline at 37°C. The approach described above allows determination of the LD_{50} and the minimum lethal dose (MLD) which is the lowest dose capable of killing two mice in the two mice group (or one mouse in the one-mouse group) after 24 hr. The toxin content is expressed in terms of Mouse Units gram (MUg) where 1MUg is 1g of mouse killed by the MLD (or the LD_{50}) expressed in g.e.f/g. The toxin concentration is expressed in MUg per gram of flesh (MUg/g.e.f) which is the reciprocal of MLD (or LD_{50}).

The suitability of fish for consumption may be controlled by mouse bioassay using 50g of flesh (Table 1). Quantitative and semi-quantitative conclusions are prescnted. Two hours are needed to prepare LR. If dissolution of LR is difficult, 0.1 ml of ethanol can be added per 1.9ml of Tween solution. A negative control is run using two mice injected with a blank solution (without LR). Non-toxic fish extracts give negative results (they elicit no symptoms).

Acute toxicity may be determined in two steps (Table 2) with a minimum of animals (Lorke et al.,1983). For this method 200g of flesh should be extracted and four hours are needed to prepare the LR. In the initial investigation, which requires an amount of extract corresponding to about 100g of flesh and 6 mice, an approximate range of doses producing the toxic effects is established. Normally this initial investigation would include doses used in the control of fish for consumption method i.e. injection of 1g.e.f/g as a first step; in

TABLE 2. Determination of the acute toxicity of LR, prepared from 200g of flesh, in two steps.

1st step EXPERIMENTAL				2nd step				RESULTS			
Doses d_1 in g.e.f./g of mouse Lethality[a] in the first test (5 possibilities)			Approximate deduced toxin concentration in MUg/g.e.f.	Doses d_2 in g.e.f./g chosen for the second test (2 mice per dose)				Corresponding toxin concentration in MUg/g.e.f. according to the (MLD)			
1[c]	0.49	0.24									
0/2	0/2	0/2	<1	[2][b]	1.7	[1.4][b]		0.5 (2.0)	0.59 (1.7)	0.71 (1.4)	-
1/2	0/2	0/2	>0.5 and <1	1.4	1.2	[0.84]		0.71 (1.4)	0.83 (1.2)	-	-
2/2	0 or 1/2	0/2	≥1 and <2	0.84	0.7	0.59		1.00 (1.0)	1.19 (0.84)	1.43 (0.70)	1.70 (0.59)
2/2	2/2	0 or 1/2	≥2 and <4	0.41	0.35	0.29		2.04 (0.49)	2.44 (0.41)	2.86 (0.35)	3.45 (0.29)
2/2	2/2	2/2	≥4	0.20	0.17	0.14	0.12	4.17 (0.24)	5 (0.20)	5.88 (0.17)	7.14 (0.14)

[a] Number of animals died/number of animals used
[b] One mouse per dose
[c] This dose allows control of fish for consumption.
[ ] Possible only when control of fish for consumption is used instead of the first test.

that case according to the observed lethality 0/2 or 1/2 it may be possible to bypass the 0.49 and 0.24 g.e.f/g injections (but not if a 2/2 lethality is observed). Based on these results, further specific doses are administered to a group of one mouse or two per dose depending on the predicted toxin concentration (<1 or >1MUg/g.e.f). From the results of these two tests, 6 or 7 successive levels of dosage ($d_1 + d_2$) are then assayed. For a two-mice group an LD_{50} can be calculated by the method of Weil (1952) using the equation

$$\log LD_{50} = \log D_a + \log R\,(f + 1)$$

D_a being the lower dosage level, R the geometric factor and f a value given in Tables. This permits a simple and rapid estimation of the LD_{50} with a corresponding confidence interval.

For the one animal group per dose, LD_{50} is estimated as the geometric mean of the doses for which 0/1 and 1/1 are found (Lorke et al.,1983). The minimum lethal dose can be used instead of LD_{50}.

RESULTS AND DISCUSSION

Control of Fish for Consumption

The proposed acetone method is much more economic and is as rapid as the method of Lee et al. (1987) who used methanol as the extracting agent instead of acetone for okadaic acid, a toxin chromatographically related to ciguatoxin. The methanol method is convenient only if the tissue portions to be extracted are <10g. So we prefer the procedure with acetone (Vernoux,1981) and we have been using this method since 1981.

In our method the LR yield must be below 0.15% of the flesh since the less impurities present the more marked the symptoms in mice for a given dose. Doses received by mice with this method do not exceed 1.5 mg of LR/g of mouse. Our proposed interpretation of symptomatology and lethality includes:

- the unique propensity of ciguatoxin to induce penile symptoms i.e penile cyanosis and/or transitory and incomplete erection (sometimes even reaching priapism i.e. complete and permanent erection seen following sub-lethal doses (Vernoux & Bagnis,1976; Vernoux et al.,1985). This symptom was recently confirmed by Terao et al., (1991) who pointed out the penis as a target organ for ciguatoxin.

- the symptoms in mice after i.p injection of okadaic acid (Vernoux & Moulin,1989) or fatty acids (Vernoux,1981) are different from that elicited by CTX but resemble the effects of maitotoxin, a toxin never detected in fish flesh (Yasumoto et al.,1984).

- the existence of a narrow range of doses (d - 2d) between 0% and 100% lethality (Hoffman et al.,1983; Lewis & Endcan 1984; Vernoux & Moulin,1989).

- the general observation of a minimum pathogenic dosage only 1/4 to 1/3 the LD_{50} dosage and the link between the pathogenic dosage and loss of weight (Chungue et al.,1984; Vernoux,1988).

- additional observations suggest a 5 MUg or 10 MUg dosage/g of mice if survival time is respectively about 1 hour and half an hour but it may vary considerably with fish species (Vernoux and Tahla,1989). The relationship between the mouse response and the quantity of toxin present is given in Table 1 and 2.

Acute Toxicity Determination

Extracting 200g is convenient for investigating fish for consumption and for quantifying the toxin concentration in the 0.5–7.14MUg/g.e.f range. This range is sufficiently wide to include toxin concentrations found in fish in the Australia, Pacific area or the Caribbean. There is no upper limit for the determination of toxin concentration, since the more toxic the flesh the less RL consumed in the test. Unlike the method for the control of fish for consumption, acute toxicity determination takes more than one day to conduct. Fortunately, stability of toxins in 1% Tween 60 saline is complete when samples are stored at –20°C for up to 6 months.

The geometric factor R = 1.1938 was chosen to provide a closely spaced series of dosage levels. This geometrical series of numbers increases successively by a constant power of 10 as already mentioned above. Furthermore, as $(1.1938)^4 = 2.0$ another geometric factor R = 2 can be used and numbers of the corresponding series are therefore included in the first one. The two-step method shown in Table 2 was developed using these series. Since the first one is a closely spaced series, this enhances the precision of the MLD determination. In this case we observed that the MLD values obtained with two animal groups were equivalent to the LD_{50} values obtained with four animal groups (Vernoux and Tahla,1989). This experimental correlation can be easily explained since the slope of the dose response curve for ciguatoxin is high with a narrow dose range (d–2d) between 0% and 100% lethality, thus including MLD and LD_{50} values (Vernoux and Moulin,1989). Nevertheless the two-mice group or even one-mouse group also give reliable LD_{50} values (Weil,1952; Lorke,1983). Here with the two-mice group, to calculate LD_{50} we use the method of Weil (1952) since it is easier and more rapid than the method of Litchfield & Wilcoxon (1949) and the former approach allows the confidence interval to be estimated. However, we prefer MLD determination to LD_{50} calculations since a greater accuracy is not necessary in view of the range of variation from one dose to another.

It might be thought that MLD or LD_{50} could be determined from the curve of dose (d) versus survival time (t) particularly since the test can be conducted in one day. Nevertheless this method is convenient only if the toxin concentration in flesh is ≥ 2MUg, thus limiting its applieation. The relationship is $d = LD50\ (1 + 1/t)^b$ or $d/LD50 = (1 + 1/t)^b$ i.e. number of MUg/g.e.f $= (1 + 1/t)^b$. Unfortunately b is fish-species dependent and it varies from 2 to 3 (Vernoux,1991) thus complicating the situation. So this method is of limited interest in controlling fish for consumption.

CONCLUSIONS

Presence of multiple ciguatoxins in fish flesh led us to propose here a simplified mouse ciguatoxin bioassay. Exhaustive description of the method should allow it to be use to control fish by any unspecialised hygiene laboratory. The limited quantity of flesh used (200g) is convenient for investigating fish for consumption and it allows to determine toxin concentration in all situations. The fixed method should replace the multiple mouse ciguatoxin bioassay methods for which toxicological bases are not very clear. Standardisation of the mouse strain could be realised with a known toxin having a similar physiological effect, brevetoxin for example. We hope that our proposals will gain wide acceptance, since the mouse ciguatoxin bioassay proposed here provides both a qualitative and semi-quantitative bioassay for ciguatoxins in fish.

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